Human hematopoietic (CD34+) stem cells possess high-affinity receptors for adenovirus type 11p

Ya-Fang Mei, Anna Segerman, Kristina Lindman, Per Hörnsten, Anders Wahlin, Göran Wadell

Department of Virology, Umeå University, S-901 85 Umeå, Sweden
Department of Internal Medicine, Umeå University, Umeå, Sweden

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Abstract

Gene transfer into human hematopoietic stem cells using Ad5 is inefficient due to lack of the primary receptor CAR and the secondary receptors αvβ3 integrin and αvβ5 integrin, and due to the high seroprevalence of Ad5 antibodies in most adults, resulting in diminished gene transduction. In the present study, we screened six species (species A–F) of adenovirus, displaying different tropisms for interaction with CD34+ cells, at the level of virus attachment and expression. Viral particles were biotinylated and their binding capacity was determined by FACS analysis using streptavidin–FITC. Ad11p, Ad35, and Ad3 (species B) showed high binding affinity, while Ad7, Ad11a (species B), and Ad37 (species D) displayed intermediate affinity. Virions of Ad4 (species E), Ad5 (species C), Ad31 (species A), and Ad41 (species F) hardly bound to hematopoietic progenitor cells. Using a double-labeling system, we demonstrated that adenoviruses bind to quiescent CD34+ cells. Ad11p virions showed the highest affinity among the adenoviruses detected. We further confirmed that virus fiber-specific receptors were present on the hematopoietic progenitor cell surface, because both recombinant fiber of Ad11p and specific antiserum against r fiber could block virus attachment. The ability of the adenoviruses to infect hematopoietic cells was studied by immunofluorescence staining. The adenoviruses from species B and Ad37 showed higher infectivity than Ad31, Ad5, Ad4, and Ad41. Among the studied species B adenoviruses, Ad11p manifested a superior infectivity. Thus, we have confirmed that these cells have high-affinity receptors for species B:2 human adenovirus, Ad11p, and this virus may be used as candidate vector to target therapeutic genes to hematopoietic stem cells.

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Introduction

Human hematopoietic stem cells (HSC) have the capacity to differentiate into hematopoietic cells. The hematopoietic cells include all cells in the peripheral blood such as T cells, B cells, monocytes, NK cells, dendritic cells, granulocytes, megakaryocytes, and erythrocytes. All of these specific functions of HSCs have attracted scientists to investigate hematopoietic cell-directed gene therapy (Graf, 2002). This could be used to replace a missing or damaged gene in congenital diseases, or to transfer a drug-resistance gene to normal HSCs to tolerate antitumor chemotherapy. Furthermore, it could also be applied to intracellular vaccination of, for example, HIV patients (Chirmule and Pahwa, 1996). Thus, HSCs represent an extremely important potential target for gene therapy.

There are several advantages to using human adenovirus 5 (Ad5), of species C, as vector for gene transfer to HSCs: (1) it can infect nondividing cells, e.g., quiescent stem cells; (2) it is easy to prepare a high titer of the virus; and (3) low risk for insertion mutagenesis. The first step in efficient gene transfer is effective virus binding to target cells. The cellular receptor for Ad5 is the 46-kDa coxsackievirus and adenovirus receptor (CAR) (Bergelson...
et al., 1997; Tomko et al., 1997). This primary receptor recognizes the knob on the distal end of the adenovirus fiber, which protrudes from the adenovirus capsid. Cells lacking primary virus receptors will be resistant to virus infection (Freimuth, 1996). Internalization of Ad5 into epithelial cells is mediated by an RGD sequence located on the penton base, and this can interact with the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, whereas internalization in the case of HSCs may be mediated by integrin $\alpha_m\beta_2$ (Huang et al., 1996; Wickham et al., 1993) because cells expressing integrin $\alpha_m\beta_2$ show markedly enhanced internalization. However, neither the primary receptor (CAR) nor the secondary receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins) are inefficiently expressed on the cell surface of HSCs (Chen et al., 1996).

The Ad5-based vectors have been used for purging epithelial tumor contaminations of bone marrow and peripheral blood, due to the lack of corresponding primary and secondary receptors for Ad5 on HSCs (Chen et al., 1996). It has been shown that Ad5 vectors are capable of infecting HSC cells at high doses of virus, corresponding to a MOI of at least 500 (Marini et al., 2000). Obviously, infection with high MOIs could induce cytotoxicity and immunogenicity in vivo and this approach is therefore not suitable for gene therapy. Consequently, the use of Ad5 for transduction of HSC is not feasible.

Fifty-one human adenovirus serotypes have been identified (De Jong et al., 1999). They can be divided into six species, A to F. Species B is subdivided further into B:1 and B:2 (Wadell, 1984; Wadell et al., 1980). Members of species A, B:1, C, and E cause respiratory or eye infection, species F causes intestinal infection whereas most adenoviruses of species B:2 infect the urinary tract. The different tropisms of adenoviruses are dependent on the adenovirus cell attachment protein, the fiber, which is a capsid protein strategically located at the 12 vertices of the viral capsid. The fiber knob has a critical role in virus–host cell receptor interaction and also mediates hemagglutination. The tropisms of different adenoviruses are apparently based on variability in the fiber knob. Two distantly related fiber knobs can initiate similar infections utilizing completely different primary receptors; for example, Ad5 and Ad11a both infect the respiratory tract, with Ad5 binding to CAR while Ad11a attaches to another unknown protein. On the other hand, closely related fiber knobs can initiate infections leading to completely different diseases: Ad11p and Ad11a genomic variants belong to the same serotype, with Ad11p infecting the urinary tract and Ad11a being associated with respiratory infection (Li et al., 1991). Ad11p and Ad35 virions of species B:2 bind efficiently to and infect committed hematopoietic cell lines (Segerman et al., 2000; Shayakhmetov et al., 2000). There is, however, limited information concerning the interaction between HSC cells and adenoviruses from the six species. We have therefore investigated the binding affinity and infectivity of representative adenovirus serotypes to select candidate adenoviruses as vectors for gene transfer to hematopoietic progenitor cells.

**Results**

**Evaluation of biotin-bound adenovirus by SDS-PAGE**

The extent of biotinylation of virions from the different serotypes was estimated by SDS-PAGE using silver staining and Western blot. The hexon polypeptides showed similar concentrations both in silver-stained gels and in Western blot, where biotinylated hexons of each adenovirus species were detected by streptavidin–HRP and then ECL. The hexon proteins from each species vary slightly in size but comparable amounts of the biotinylated hexons were detected in both protein staining gels and Western blot (Fig. 1). The results indicated that measurements of viral protein and biotin bound were comparable and sufficiently reliable for quantification of virus binding.

**Adenoviruses belonging to the six species manifested different binding capacity for hematopoietic progenitor cells**

The characteristics of the hematopoietic progenitor cell surface components to which adenoviruses from the six species bound are shown in Fig. 1. The affinity of biotinylated Ad11p virions was used as negative control: NC. The position of hexon protein is indicated by an arrow.
species attach were investigated using a virus-binding assay. Cells were freshly prepared using immunomagnetic beads (Dynal, AS, Oslo, Norway) coupled with mouse anti-human CD34⁺ monoclonal antibody and incubated at 4°C with biotinylated viruses and then with streptavidin–FITC, and the amount of virus bound to cell surface was quantitated by FACS analysis. The binding capacities of adenoviruses with various tropisms to CD34⁺ progenitor cells from bone marrow are illustrated in Fig. 2. The fluorescence histogram of adenoviruses shows various binding affinities for CD34⁺ cells, which can be classified into three groups: high, intermediate, and low or undetectable affinity. Virions of Ad3, Ad11p, and Ad35 displayed high binding affinity, i.e., with 1 μg of labeled virions, more than 90% of cells were positive in the case of Ad11p, whereas about 50% were labeled in the case of both Ad3 and Ad35 virions. Ad7, Ad11a, and Ad37 manifested an intermediate affinity for CD34⁺ cells, i.e., 20% of the CD34⁺ cells scored positive when 1 μg of virions were added. Virions of Ad31, Ad5, Ad4, and Ad41 showed no detectable affinity for the CD34⁺ cells.

By increasing the amounts of virions from 1 to 10 μg per million cells, the attachment of Ad3, Ad11p, and Ad35 increased in a dose-dependent manner. More than 95% of the cells scored positive when they were exposed to 1 μg of labeled Ad11p virions. Sixty-five percent and 80% of cells bound Ad3, whereas 75% and 90% of the cells bound Ad35 after the addition of 3 and 10 μg of the respective virions. The proportion of positive cells also increased when CD34⁺ cells were exposed to increasing concentrations of Ad7, Ad11a, and Ad37 virions. However, even after the amount of biotinylated virions had been increased to 10 μg, which corresponds to 3.5 × 10¹⁰ virion particles

Fig. 2. Flow-cytometric analysis of the binding capacity of representative adenoviruses from six species to human CD34⁺ cells. One million freshly purified CD34⁺ cells were tested for their ability to bind 1 μg of biotinylated adenoviruses with various tropisms. Detection of virus binding was carried out with streptavidin–FITC and control samples were incubated without virions.
(10 pg/cell), the portion of CD34+ cells that bound Ad4, Ad5, Ad31, and Ad41 remained unchanged. Thus, less than 5% of the CD34+ cells showed affinity for the representative adenoviruses belonging to species A, C, E, and F (Fig. 3, Table 1).

**Identification of CD34+ cells bound by some adenovirus serotypes**

A double-labeling method, whereby CD34+ cells were labeled by fluorescence staining and virions were biotinylated and detected by RPE staining, was used to ascertain that the hematopoietic cells with affinity for adenoviruses were indeed CD34+ cells. Freshly purified CD34+ cells were bound to biotinylated virions at a concentration of 0.1 μg of Ad11p per a million CD34+ cells. Under these conditions, 53.2% of the cells were labeled by both the virions and CD34+ marker (double labeled). With the same quantity of Ad11a and Ad37 virions, only 12.8% and 17.1% of the cells, respectively, were double labeled. By increasing the quantity of Ad11p virions to 0.5 μg, the proportion of double-labeled positive cells apparently increased to 80.7%, but only reached values of 18.2% and 17.1% when the CD34+ cells were exposed to Ad11a and Ad37 virions, respectively. Thus, the cells binding adenoviruses were interpreted to represent CD34+ cells (Fig. 4).

**Blocking by Ad11p r fiber and Ad11p r fiber antibody demonstrated that the specific binding of virus to CD34+ cells is mediated by the adenovirus fiber**

The interaction between the virus capsid and the host cell receptor would be expected to be mediated by adenovirus fiber. To confirm this, seven different concentrations of Ad11p r fiber were mixed with the CD34+ cells at 4 °C for 1 h, followed by incubation with 35S-labeled Ad11p virions. The proportion of cells that could bind Ad11p virions was determined. A low quantity of Ad11p r fiber (1 ng) blocked 70% of the virus binding (Fig. 5). By increasing the concentration of the Ad11p r fiber, the efficacy of the blocking improved, with 3.6 μg of r fiber blocking 98.3% of Ad11p virions from binding to CD34+ cells (Fig. 5).

The ability of hyperimmune serum against Ad11p r fiber to block the attachment of Ad11p virions to CD34+ cells was also tested. Serial dilutions of rabbit hyperimmune serum against Ad11p r fiber were preincubated with 106 35S-labeled Ad11p virions and then incubated with CD34+ cells. The control was incubated with buffer devoid of Ad11p r fiber antiserum. At a dilution of the antiserum as high as 1:12,800, 50% of the binding was blocked. At higher concentrations of the antiserum (1:3200 and 1:800), the inhibition reached 70% and 80%, respectively (Fig. 6).

**The binding and replication of Ad11p virions detected by immunofluorescence microscopy**

Adenovirus replication studies were performed in HSC cells with the 10 adenoviruses described above. HSC cells were infected with the various viruses at a high dose (2 pg, equivalent to 7 × 103 physical particles per cell). The infected cells were treated with the adenovirus hexon-specific monoclonal antibody against all serotypes of adenovirus, respectively. Then, the cells were stained with streptavidin–FITC alone before analysis using flow cytometry.

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### Table 1

<table>
<thead>
<tr>
<th>Serotype or genome type</th>
<th>Species</th>
<th>Tropisms (or symptoms)</th>
<th>Binding affinity to CD34+ stem cells</th>
</tr>
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<tbody>
<tr>
<td>Ad11p</td>
<td>B:2</td>
<td>Kidney</td>
<td>+++</td>
</tr>
<tr>
<td>Ad35p</td>
<td>B:2</td>
<td>Kidney</td>
<td>++</td>
</tr>
<tr>
<td>Ad11a</td>
<td>B:2</td>
<td>Respiratory tract</td>
<td>+</td>
</tr>
<tr>
<td>Ad3p</td>
<td>B:1</td>
<td>Respiratory tract</td>
<td>++</td>
</tr>
<tr>
<td>Ad7p</td>
<td>B:1</td>
<td>Respiratory tract</td>
<td>+</td>
</tr>
<tr>
<td>Ad37</td>
<td>D</td>
<td>Eye and genital tract</td>
<td>+</td>
</tr>
<tr>
<td>Ad31p</td>
<td>A</td>
<td>Enteric tract infection</td>
<td>–</td>
</tr>
<tr>
<td>Ad5p</td>
<td>C</td>
<td>Respiratory and lymphoid tissue</td>
<td>–</td>
</tr>
<tr>
<td>Ad4p</td>
<td>E</td>
<td>Eye and respiratory tract</td>
<td>–</td>
</tr>
<tr>
<td>Ad41p</td>
<td>F</td>
<td>Enteric tract infection</td>
<td>(diarrhea)</td>
</tr>
</tbody>
</table>

Note. “p” means that the adenovirus prototype was used.

*++*, with 1 μg of virus, 80–90% CD34+ cells scored positive. **+, with 1 μg of virus, 40–50% CD34+ cells scored positive. +, with 1 μg of virus, 10–30% CD34+ cells scored positive. –-, with 1 μg of virus, less 5% CD34+ cells scored positive.
Ad35 was observed at 24 h p.i. Cells infected with Ad3 and Ad7 showed clear hexon-positivity at 48 h p.i. A cytolytic effect was only observed in the cells infected with Ad11p and Ad35. In comparison to species B adenoviruses, Ad37 with a corresponding dose of inoculum could infect HSC, but the replication of Ad5, Ad4, Ad31, and Ad41 was undetectable even when the time point was extended to 72 h. Thus, species D (Ad37) and species B adenoviruses could infect the hematopoietic cells better than adenoviruses from other species. However, Ad11p replicated more efficiently than all other studied adenoviruses in hematopoietic cells (Fig. 7).

**Discussion**

The transfer of new genetic material to hematopoietic progenitor cells and expression of the gene product in

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Fig. 4. Two-color flow cytometry analysis of the interaction between adenoviruses and CD34+ cells. Freshly purified CD34+ cells were incubated with biotinylated adenoviruses at 4°C for 30 min, the cells were washed once with PBS–FCS–NaN3 buffer, and then simultaneously incubated with RPE–avidin and MAb–FITC specific for CD34+ cells, and subjected to flow cytometric analysis. The cells bound by adenoviruses showed red fluorescence (upper left). Green fluorescence indicates CD34+ cells (lower right). Double-labeled cells are localized in the upper right window. The double-staining results indicated that Ad11p binds strongly to hematopoietic stem cells, and that Ad11a and Ad37 also show appreciable binding.

Fig. 5. Measurement of the capacity of purified recombinant fiber (rfiber) to block the attachment of Ad11p virions to CD34+ cells, by flow cytometry. The Ad11p rfibers were incubated with CD34+ cells at the indicated concentrations for 30 min at 4°C to allow binding to cellular receptors. Biotinylated infectious Ad11p virions (3 pg/cell, which is equivalent to 10^9 particles/cell) were added, and the incubation was continued for 30 min to allow virus adsorption. Staining and flow cytometry analysis was performed as described in Materials and methods.
progeny of various lineages is an exciting approach to the treatment of congenital and acquired human diseases. However, it is becoming clear that the commonly used retrovirus-based or adenovirus-based vectors are associated with certain as yet unresolved problems. Retroviruses cannot transduce human quiescent cells and the commonly used Ad5 induces a CTL response and also shows a high prevalence of this serotype (D’Ambrosio et al., 1982; Routes et al., 1991; Vogels et al., 2003). Moreover, hematopoietic progenitor cells lack the expression of appropriate receptors for both viruses (Chen et al., 1996; Crystal, 1995). Thus, it is pertinent to explore alternative vector candidates that exhibit a high degree of binding to CD34+ cells and are neither pathogenic nor have a high prevalence. In this paper, we have studied adenovirus binding capacity and replication, and have demonstrated that Ad11p, Ad35, and Ad3 of species B show high binding efficiency in their interaction with human hematopoietic stem cells.

Adenoviruses belonging to the different species manifest various tropisms; also, the binding capacities of different adenoviruses for CD34+ cells vary. Thus, the FACS experiments indicated markedly different binding capacities for HSC cells when representatives of the six species were compared. The existence of high-affinity receptors for Ad11p and Ad35 virions on quiescent CD34+ cells was demonstrated, whereas virions of Ad31, Ad4p, Ad5, Ad41 showed no demonstrable affinity for CD34+ cells. Ad37, Ad11a, Ad3, and Ad7 virions showed intermediate binding capacity. The commonly used Ad5 vector showed a minimal binding affinity: to less than 5% of HSC cells even with a high dose of virus (10 pg/cell). Ad5 attaches to the cell surface through the coxsackie-adenovirus receptor (CAR), and is internalized via α1β3 and α1β5 integrins. Less than 3–5% of CD34+ cells were integrin receptors, α1β3 and α1β5 positive (Chen et al., 1996). This absence or low degree of CAR expression of cellular receptors for the Ad5 fiber protein on CD34+ cells...
explains why these cells are almost completely resistant to binding to HSCs. Ad11p showed an unsurpassed high binding affinity, and Ad35 and Ad3 showed a somewhat lower binding affinity for human HSC cells. Measurement of the infectivity of the studied adenoviruses revealed that the species B adenoviruses and Ad37 can, but Ad31, Ad5, and 4 and Ad41 cannot, infect hematopoietic cells efficiently. Among them, Ad11p, Ad3, and Ad35 manifest a higher infectivity to hematopoietic cells. Our data agree with earlier studies showing that the Ad5/35FK and Ad35 vector was capable of transfecting CD34+ cells (Sakurai et al., 2003; Shayakhmetov et al., 2000). However, whether the interaction between these two adenoviruses and HSC affects cell differentiation is still unknown. Furthermore, the binding, internalization, and replication of adenoviruses in primary cells of hematopoietic origin, such as T cells, B cells, and monocytes remain to be investigated.

We demonstrated that Ad11p and Ad35 share the same cellular receptors, because the binding kinetics of the two viruses to epithelial cells were similar and both virions and fibers can block each other in a reciprocal fashion (Mei et al., 1998), even though the two fiber knobs only share 50% amino acid homology (Mei and Wadell, 1995). Ad11p and Ad35 can specifically attach to HSC cells and replicate in these cells. Ad11p and Ad35 also show a tropism for kidney and are often isolated from bone marrow or kidney transplant patients. The mechanisms of transmission are unknown. Adenoviruses with a tropism for kidney also display affinity for hematopoietic progenitor cells. Interestingly, in terms of embryonic development, both kidney and hematopoietic cells are of mesodermal origin. The distribution of receptors for Ad11p could thus be related somehow to embryonic stem cell development. Our findings suggest that receptors for species B:2 adenoviruses are present on the surfaces of hematopoietic stem cells. Ad11p and Ad35 internalization in HSC cells may not be mediated by αvβ3 and αvβ5 integrins, which ordinarily leads to endocytosis via the formation of clathrin-coated pits. In support of this model, the attachment of adenoviruses to epithelial cells could not be blocked by anti-integrin antibodies. Despite the fact that quiescent CD34+ cells express very low amounts of αv integrins, Ad11p binding to and infection of hematopoietic cells was highly efficient. These results imply that there is a possibility that Ad11p internalizes via a non-integrin adhesion protein.

Although the two closely related adenovirus genotypes, Ad11p and Ad11a, show high and intermediate binding capacity for CD34+ cells, respectively, they have different tropisms: one for the urinary tract and the other for the respiratory tract. They also show a completely different hemagglutination pattern; Ad11p can, but Ad11a cannot, aggregate monkey erythrocytes (Mei and Wadell, 1996). The fiber knobs, suggested mediators of attachment to the host cell receptors, share 92% identity.

Only 10 amino acid residues differ in the fiber knobs of Ad11p and Ad11a. Some of these contribute to the pronounced differences in the binding capacity to CD34+ cells. The discrepant amino acids between the two fiber knobs of the viruses contribute the various binding affinity to hematopoietic stem cells. Reciprocal competition experiments between Ad11p and Ad11a on epithelial A549 cells have indicated that Ad11p can block Ad11a binding to some extent, while Ad11a cannot block Ad11p at all (Mei et al., 1998). Thus, there appear to be two heterogeneous receptors attached by Ad11p and Ad11a, one that is common and one receptor specifically associated with kidney tropism. These receptors have been designated sBAR and sB2AR in the recent publications (Segerman et al., 2000, 2003a). Attachment of the two adenoviruses to HSCs implies that either only the common receptor (sBAR), or both receptors (sBAR and sB2AR), for Ad11p and Ad11a contribute to attachment of the viruses to HSCs. Recently, it has been demonstrated that Ad11p binds CD46 as a primary receptor (Segerman et al., 2003b), which is expressed on all peripheral blood cells and platelets, but not on erythrocytes. CD46 is also present on fibroblasts, endothelial cells, and epithelial cells. Thus, Ad11p binds to and internalizes in CD34+ cells but the mechanisms remain to be investigated.

We have reported that both Ad4 (species E) and Ad11p (species B:2) manifested superior binding and infectivity in epithelial A549 and 293 cells, and they produced the highest amounts of viral structural proteins in infected neural cells (Mei et al., 2002; Skog et al., 2002). However, the present study has demonstrated a distinct difference in binding affinity between Ad11p and Ad4 for CD34+ cells: very high and undetectable, respectively. In comparison with these two adenovirus serotypes, the commonly used Ad5 showed lower binding affinity to epithelial cells and no detectable binding affinity to CD34+ cells. Ad4 causes conjunctivitis and respiratory infection, and was shown to interact with a soluble CAR preparation in a dot blot assay (Roelvink et al., 1998). The biological properties of Ad4 in various cells suggest that this virus has a superior CAR-binding capacity or uses an unknown co-receptor in addition to CAR.

Chimeric fibers based on Ad5 vector have been successfully constructed and the species B (Ad3) hexon gene has also been placed in the Ad5 genome (Havenga et al., 2002; Wu et al., 2002). Although this can improve gene transfer and overcome the problem of high seroprevalence, it is difficult to produce high amounts of chimeric vectors.Gene transduction efficiency or virus infectivity was dependent on the properties of the ligand in the fiber knob. The number of receptors on the host cells are also one of the most important factors. Low receptor concentration or incompatible fiber will greatly impair gene transduction or infectivity. Ad11 virions showed a high degree of binding to and replication in epithelial, kidney, and bladder cells, but also have a high binding capacity for brain tumor cell lines (Skog et al., 2002). Ad11p virions bind remarkably well to endothelial cells and tumor cells originating from the lung, breast, liver, and prostate (Zhang et al., 2003). Both Ad11p and Ad35
show a high binding affinity for hematopoietic cell lines (Segerman et al., 2000).

In summary, Ad11p virions were shown to bind most efficiently to CD34+ cells. Specific attachment to the primary receptors on the CD34+ cells was mediated by the adenovirus fiber, as this could be completely blocked by fiber or specific antibodies to the fiber of Ad11p and Ad35. Thus, Ad11p appears to be a potential gene transfer vector for hematopoietic CD34+ cells.

Materials and methods

Virus strains and virion purification

Ten human adenovirus serotypes or genome types including Ad31 (species A), Ad3, Ad7, Ad11p, Ad11a, Ad35 (all of species B), Ad5 (species C), Ad37 (species D), Ad4 (species E), and Ad41 (species F) were selected to represent all six species and different tropisms. The adenoviruses were represented by the prototypes, with the exception of Ad11a. All viruses were propagated on A549 cells (human oat cell lung carcinoma) and purified by ultracentrifugation in CsCl gradients (Mei et al., 1998). Determination of virus particle titer was accomplished spectrophotometrically by using a conversion factor of 280 μg being equivalent to 1 × 10\(^{12}\) virus particles per absorbance unit at 260 minus 330 nm, as previously reported (Greber et al., 1999).

Biotinylation of virions

One hundred microliters of N-hydroxysuccinimidobiotin (Sigma Chemical Co., St. Louis, MO) was added to 1 ml of the virions (1–5 mg/ml) in labeling buffer (50 mM NaHCO\(_3\), 2 mM MgCl\(_2\), 135 mM NaCl, pH8.8). The virions were mixed with biotin overnight by shaking at 4°C in the dark (Inghirami et al., 1988). The solution was then passed through a NAP-10 column (Pharmacia) equilibrated with phosphate-buffered saline (PBS), and unbound biotin was then removed. The concentration of biotinylated virions was determined by spectrophotometry. Glycerol was added as 10% of the total volume and the virions were aliquoted in small volumes and stored at –80°C until use.

Evaluation of biotinylated adenoviruses by SDS-PAGE and Western blotting

Biotinylated virion particles (2.8 μg) from each species were suspended in lysis buffer and loaded onto a 12% SDS-polyacrylamide gel. Two gels with the same samples loaded were run in parallel. After electrophoresis, standard silver staining was carried out for one gel, whereas the other gel was processed for Western blot. After the transfer procedure, the membrane was blocked with PBS–Tween–milk (0.05% Tween 20, 5% nonfat dry milk) for 1 h at RT and washed five times with PBS–Tween. The biotinylated virion capsid proteins were detected by streptavidin–HRP (1:3000 dilution; DAKO A/S, Denmark) and then ECL.

Isotope labeling of adenovirus

\(^{35}\)S-labeled adenovirus was produced in A549 cells as described by Greber et al. (1993), with minor modifications. A549 monolayer cultures (1 × 10\(^7\) cells) in 175-cm\(^2\) flasks were infected with 26 μg Ad11p virus (7.2 × 10\(^8\) particles per cell, 100 TCID\(_{50}\), where 1 TCID = 72 particles) in 6 ml DMEM containing penicillin and streptomycin and 0.02% bovine serum albumin (BSA), and incubated for 90 min at 37°C. Unbound virions were washed off and the cells were incubated in 50 ml DMEM containing 5% FCS per flask for 20 h. The cells were then rinsed once with methionine–cysteine free DMEM and maintained in 8 ml of the same medium with 5% FCS for 2 h at 37°C to deplete endogenous methionine and cysteine. The cells were then labeled with 1.75 mCi (64.8 mBq)/bottle of Tran \(^{35}\)S-label™ methionine and cysteine (ICN Pharmaceuticals Inc., Irvine, CA) for 1 h; 2 mM unlabeled cysteine was then added and the cells were maintained for a further 7 h, after which 2 mM unlabeled methionine was added. At 24 h postinfection, the same amounts of unlabeled cysteine and methionine were again added to the cells. The viruses were harvested and purified 72 h postinfection. The \(^{35}\)S-labeled viruses were purified and quantified as described above. They were aliquoted and stored at −70°C until use.

Hematopoietic progenitor cells

Fresh hematopoietic progenitor cells were selected using immunomagnetic beads (Dynabeads M-450) (Dynal) coated with an anti-CD34+ monoclonal antibody, according to the manufacturer’s instructions. Detachment of the CD34+ cells was achieved by using DetachBead (Dynal). The purified CD34+ cells were either used directly in the virus binding experiments or were cultivated. Briefly, the cells were seeded in 24-well culture plates with 2 ml long-term culture (LTC) medium containing 12.5% fetal calf serum (FCS) and 12.5% HS (horse serum; Gibco BRL, Gaithersburg, MD), supplemented with 10\(^{-6}\) M hydrocortisone, 10 ng/ml interleukin-3 (IL-3), 5 ng/ml interleukin-6 (IL-6), and 2 ng/ml stem cell factor (SCF). All three factors were bought from GENZYME Diagnostics (Cambridge, MA) and were used to stimulate hematopoietic progenitor cell expansion (Fischer et al., 1997). The purity of CD34+ preparations was verified by flow cytometry and was consistently greater than 95%.

Binding experiments

Biotinylated adenovirions (1, 3, or 10 μg) was incubated with 10\(^6\) freshly purified CD34+ cells for 30 min on ice. The cells were washed with PBS containing 2% FCS and 0.01%
NaNO₃ (PBS–FCS–NaNO₃) and pelleted by centrifugation at 1000 rpm, followed by addition of streptavidin–FITC (1:100 dilution) and mixing at 4°C for 30 min. The cells were again washed as described above but the buffer now also contained 1 mg/ml propidium iodide (PI). The amount of virus bound to the cell surface was quantitated by a FACSscan (Becton Dickinson) flow cytometer and then analyzed using the LYSYSII software program (Becton Dickinson).

Double-labeling experiments

To confirm that adenoviruses can specifically bind to human hematopoietic progenitor cells (CD34⁺), a double-labeling method was used. One million freshly selected CD34⁺ cells were washed with PBS–BSA–NaNO₃ buffer once, incubated with 0.1, 0.5, 1.5, 3.0, 6.0, and 12.0 µg of biotinylated Ad11p, Ad11a, and Ad37 with mixing at 4°C for 30 min. The cells were washed once with PBS buffer as above and pelleted by centrifugation at 1000 rpm; then 50 µl (1:50 dilution) of streptavidin conjugated with R-phycocerythrin (RPE) (DAKO) and 50 µl (1:50 dilution) of FITC-labeled mouse monoclonal antibody against human CD34⁺ marker were added. The cells were incubated at 4°C for 30 min and washed again as described above. The cells were subsequently transferred to a Falcon 2052 tube in 300 µl of the buffer. The amount of virus bound to the CD34⁺ cells was measured in by FACS analysis.

Blocking experiments using Ad11p recombinant fiber protein

We have constructed, expressed, and purified recombinant Ad11p fiber protein. This fiber protein has been identified as a trimer by native PAGE and native epitopes have been demonstrated by Western blot (Mei et al., 1998). We used rfiber to block virus binding to CD34⁺ cells with a view to ascertaining whether this binding is specific for fiber receptors. Quantities of Ad11p rfiber from 1, 2, 10, 20, 100, 200, and 3600 ng were added to freshly prepared CD34⁺ cells (5 × 10⁴) and incubated at 4°C for 1 h. The cells were incubated with 35S-labeled Ad11p virions (10⁵/cell) for another hour at 4°C and then washed. Cells bound to virions were separated from unbound virions by centrifugation, and both fractions were transferred separately into scintillation tubes and measured in a liquid scintillation (Wallac 1409). The control experiment was performed in the absence of anti-rfiber serum.

Indirect immunofluorescence microscopy

Three-hundred thousand CD34⁺ cells per well were seeded in 24-well plates with LTC medium and inoculated at a ratio of 2 pg virions/cell of Ad31, Ad3, Ad7, Ad11p, Ad11a, Ad35, Ad5, Ad37, Ad4, and Ad41, respectively. For negative control, no virus was added to the cells. After culture at 37°C for 24, 48, 72 h, the infected cells and noninfected cells were harvested and centrifuged. The cell pellets were washed twice in PBS and allowed to dry at room temperature. The cells were fixed in 100% cold methanol at 4°C for 10 min and incubated for 1 h at 37°C with a mouse anti-adenovirus hexon antibody that was specific for all serotypes of adenoviruses and purchased from Chemicon International (catalog no MAB8052) and diluted 1:200 in PBS containing 2% BSA (PBS–BSA). The cells were then washed with PBS three times, each time for 5 min and incubated for 30 min at 37°C with polyclonal rabbit anti-mouse Immunoglobulins conjugated with FITC (catalog no. F0313, DAKO) diluted 1:30 in PBS–BSA. Finally, the slides were washed as described before, mounted in Flourescence Mounting Medium (DAKO) and examined in a Zeiss fluorescence microscope (Axioskop 2) at a final magnification of 400×, and photographed with a digital Zeiss AxioCam camera.

References


